TRYPANOSOMA CRUZI: OBSERVATIONS ON ENTRY, DEVELOPMENT, RELEASE AND ULTRASTRUCTURE OF PARASITES GROWN IN CELL CULTURES[1]

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Abstract. *T. cruzi* epimastigotes penetrated cells in culture by a process which involved entanglement of the parasite's flagellum with cellular filopodia. Development of trypomastigotes from intracellular amastigotes appeared to involve a transformation through an epimastigote-like form. Release of trypomastigotes was usually by cell rupture but occasionally trypomastigotes in vacuoles were released from cells without cell rupture. The parasites were examined with the scanning and transmission electron microscopes and three-dimensional views of the organisms were obtained by the scanning and carbon replica techniques. The texture of the outer surface of the limiting membrane of the scanned trypomastigote appeared fairly smooth to granular. Granulations were more evident in the carbon-replicated parasites than in scanned ones. Many amastigotes produced in cell cultures have short flagella. Freeze-cleaved trypomastigotes showed the two lamellae of the unit membrane and a surface coat. The morphology of the outer face of the surface coat and of each face of the lamellae of the unit membrane appeared smooth whereas the inner faces of the cytoplasmic membranes and the flagellar unit membrane were granular. The granules of the former, however, were morphologically distinct from those of the latter. A common outer layer or surface coat, which enveloped both the parasite's body and its flagellum, was evident. When this layer was cleaved away, the flagellar pocket was exposed. The common layer appeared to bind the flagellum to the parasite's body.

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Trypanosoma cruzi, the causative agent of Chagas' disease, is easy to isolate and cultivate in a variety of culture media. The bulk of the flagellates in these media are in the epimastigote form and are similar to those found in the intestine of the reduviid insect vector (Bishop, 1967). In the blood of the mammalian host the parasite exists as trypomastigotes and it is possible to use mammals as a source of trypomastigotes for study. Camargo *et al* (1970) for example, collected 4 x 10^{10} trypomastigotes per liter of blood of adult dogs given an immunosuppressive drug. Developing chick embryos and cell cultures may also be used for production of trypomastigotes. Pipkin (1960) reviewed the literature on this subject and concluded that the limited success that most workers have had with chick embryos for cultivation of *T. cruzi* did not justify their use on a large scale.

A sizeable amount of information has been accumulated concerning the development of *T. cruzi* in cell culture since Kofoid *et al* (1935) first published on the subject. Recent works on cultivation of *T. cruzi* in cell cultures are those of Neva *et al* (1961), Trejos *et al* (1963), Deane and Kloetzel (1969), Rodriguez and Marinkelle (1970), and Dvorak and Hyde (1973a; 1973b). These investigators indicate that cell culture is valuable for production of trypomastigotes of *T. cruzi*, and provide much information about growth and differentiation of *T. cruzi* in cell cultures.

Romana (1955) described two ways by which trypomastigotes in cultures transform into amastigotes: "fusiform regression" and "orbicular regression". In the former the trypomastigote gradually be-

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comes shorter and broader with forward migration of the kinetoplast and loss of the flagellum until the organism assumes the amastigote form. The latter process involves the organism folding upon itself to become an amastigote. The amastigote form of the parasite divides repeatedly by binary fission and, as the number of intracellular organisms increases, the host cells enlarge. The amastigotes then develop flagella and become sphaeromastigotes. The sphaeromastigotes elongate to form epimastigotes. Stumpy trypomastigotes are then produced as a result of a backward migration of the kinetoplast and the flagellar apparatus. Stumpy trypomastigotes change first into slender and finally into typical C-shaped broad trypomastigotes before the rupture of the infected cells (Petana, 1971). A similar process of transformation of amastigotes into trypomastigotes, called elongation, has long been described from cell culture studies (Kofoid *et al*, 1935; Meyer and Oliveira, 1948; Romana, 1955; Bock *et al*, 1959).

The process of "elongation" (Rodriguez and Marinkelle, 1970) or "fusiform progression" (Romana, 1955) is widely accepted as a means of production of trypomastigotes from amastigotes. However, it has been challenged by several workers, and two alternative processes have been suggested. In the "direct progression" proposed by Wood, and cited by Silva (1959), Lamy (1971) and Dvorak and Hyde (1973a), the trypomastigote is produced by the direct elongation of the amastigote without the formation of the intermediate epimastigote. In the third process, described by Romana (1955), Silva (1959), Tang (1953), Rodriguez and Marinkelle (1970), and Elkeles (1960), the intermediate epimastigote is also not produced. By a process of unfolding (Rodriguez and Marinkelle, 1970) or "orbicular progression" (Romana, 1955), the rounded amastigote develops a flagellum and becomes a sphaeromastigote. A vacuole develops in the body of the latter stage until a trypomastigote is formed by unfolding of the body without movement of the kinetoplast. Some workers distinguished between the process of fusiform progression and the process of oribicular progression and were of the opinion that the development of the amastigote into a trypomastigote proceeds along either of the two processes (Romana, 1955; Silva, 1959; Brener, 1971). Furthermore, Silva (1959) recognized two morphologically different amastigotes, rounded and fusiform. The former develop directly into trypomastigotes by unfolding and the fusiform amastigotes transform by a process of elongation. In the present study we report observations on the entry of the parasites into cells in culture, the parasites' development in the cells, their release and their structure.

MATERIALS AND METHODS

Trypanosoma cruzi strain and its maintenance

The strain of the parasite described by Seed *et al* (1973) was used in this study. It was maintained at 26°C in Senekjie's medium (1943) with an overlay of Locke's solution and was routinely transferred every 14 days. The parasites observed were primarily epimastigotes with a small percentage of metacyclic trypomastigotes, some cultures maintained 35 days developed approximately 12% metacyclic trypomastigotes.

Cultivation of the parasite in cell culture

Production of trypomastigotes or amastigotes and observations on penetration and development of the parasites was done with confluent monolayers of Hep-2 of HeLa cells on coverslips in Leighton tubes, or 16 ounce prescription bottles infected with *T. cruzi* epimastigotes. On the day of infection the cultures were fed with fresh tissue culture medium (TCM) consisting of Joklik's modified minimum essential medium (Grand Island Biol. Co.) with 10% heat inactivated calf serum. Each culture was inoculated with organisms contained in the liquid phase of a 14-day old Senekjie's biphasic culture. At least 98% of the organisms in the cultures were epimastigotes. The infected cultures were incubated at 32.5°C or 37°C and were refed at 3 or 6 day intervals during study. Cultures used to produce trypomastigotes for morphological study were incubated at 32.5°C and were refed on the 3rd and 9th days of incubation and then once every 6 days. On the 9th day of incubation, and at the time of each subsequent feeding, parasites in the medium were counted in a hemocytometer and a smear was made for differential counts after Giemsa Stain. Aliquots of the organisms harvested from cell cultures which contained about 90% trypomastigotes, and cells and organisms grown on coverslips in cultures were processed for carbon replication, scanning electron microscopy, or freeze-etching by the techniques outlined by Seed *et al* (1973). Specimens were examined by either the Phillips EM 300 transmission electron microscope (TEM) or a Cambridge scanning electron microscope (SEM).

RESULTS AND DISCUSSION

PENETRATION, DEVELOPMENT AND RELEASE OF *T. cruzi* IN CELL CULTURES

A mixture of *T. cruzi* trypomastigotes and amastigotes were obtained from the supernatant fluid of monolayer cultures of HeLa cells grown at 32.5°C for 9 days after infection with *T. cruzi* epimastigotes. The trypomastigotes were mostly slender forms while the amastigotes were oval to round and commonly had very short free flagella (figure 1).

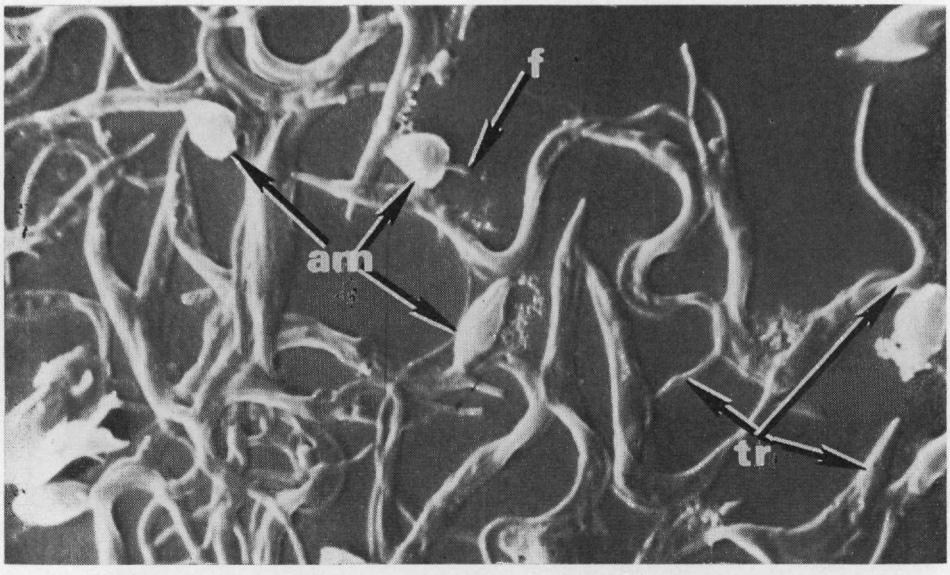
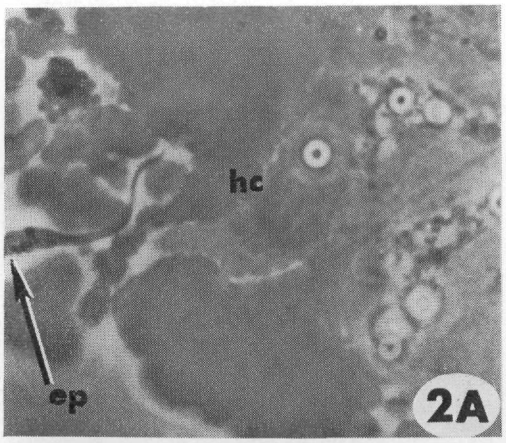
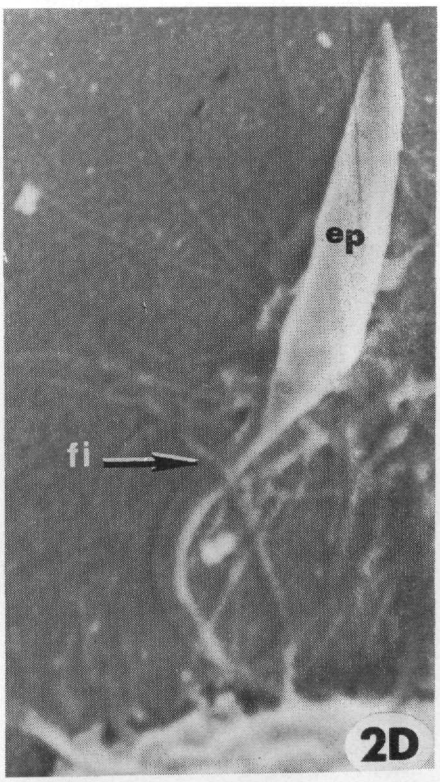


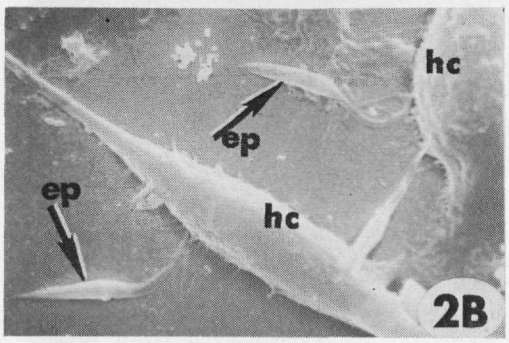
FIGURE 1. Scanning electron microscope study of trypomastigotes (tr) and amastigotes (am) harvested from HeLa cell cultures. Some of the amastigotes have a very short free flagellum (f).

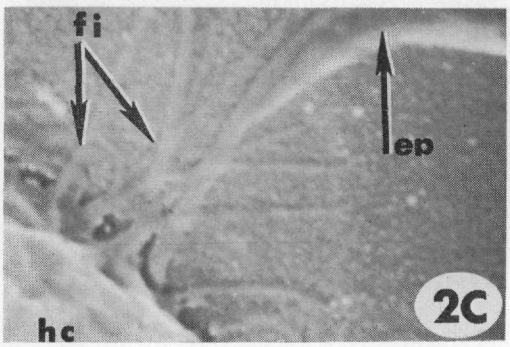
The epimastigotes used to infect the cell cultures swam in the medium and very frequently came in contact with the cells growing on the glass surface. The contact was always by their flagellum (fig. 2A) and it was not uncommon to see several organisms surrounding one cell. The parasites that contacted the cells attached themselves by the flagellar tip for up to several minutes, but ultimately most swam away. Very rarely did these organisms succeed in penetrating the cells, but when they did, the organisms were very active as they introduced themselves slowly but progressively into the cells. Entry of the epimastigotes was exclusively by their anterior end. SEM micrographs revealed that attachment to a cell occurred most often in an area where there were many cell processes (filopodia) (fig. 2B). At high magnification it can be seen that the flagellum appears to pass between the filopodia and the glass surface, and the parasite appears to be held to the cell by this entanglement (fig. 2C and D).

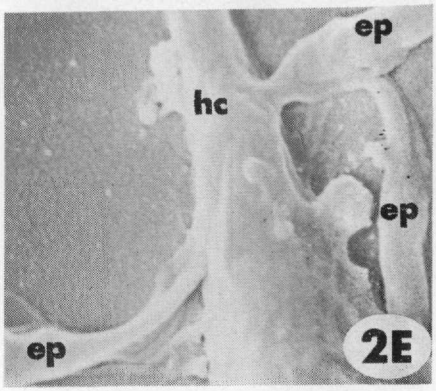
Dvorak and Schmunis (1972) studied penetration of mouse peritoneal macrophages by *T. cruzi* in culture. They suggested that epimastigote penetration was mediated by a filamentous projection at the tip of the flagellum which appeared to stick to the surfaces it contacted. They made no mention of flagellar entanglement with filopodia such as was apparent in our SEM pictures, possibly penetration into actively phagocytic cells is by a different means than penetration of cells that are not normally strongly phagocytic. It was not clear whether the penetrating organisms entered the cells by invagination of the membrane or through a hole in the cell membrane. In our SEM micrographs the parasite's limiting membrane appeared to be continuous with that of the cell. The host cell membrane was not invaginated around the entering parasites (fig. 2E).











EXPLANATION OF MICROGRAPHS 2A–E

HEp-2 cells (hc) with epimastigotes (ep) attached to them.

FIGURE 2A. Phase contrast study of an epimastogote (arrow) penetrating a host cell.

FIGURE 2B. Organisms introduced their anterior ends beneath the cells, and appeared to be attached in regions where the cell has filamentous processes, i.e. filopodia.

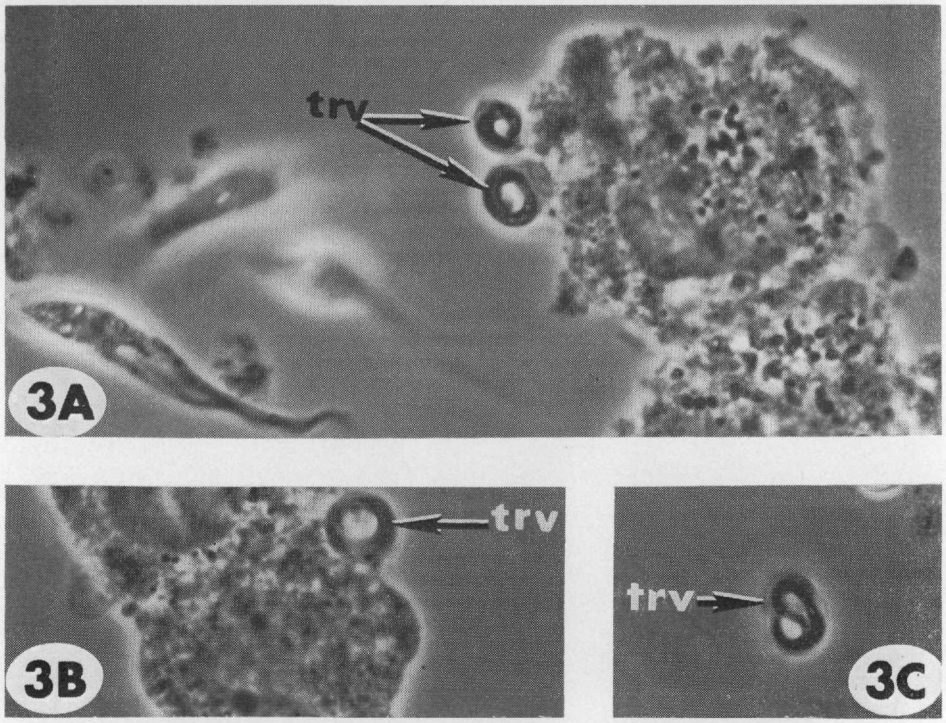
FIGURES 2C–D. The point of the attachment of the parasite is shown at high magnification and the entanglement of the parasite flagellum in filipodia (fi) processes can be seen.

FIGURE 2E. The host cells' limiting membranes appear to be continuous with that of the parasite when penetration is advanced.

Dvorak and Schmunis (1972) and Dvorak and Hyde (1973a, b) observed free movement of epimastigotes inside cells. This would suggest that the parasites are not normally inside parasitophorous vacuoles. Our scanning micrographs did not show invagination at the point of entry. This observation and the observation of free movement that Dvorak and co-workers described are compatible with a mechanism of penetration by passage through the membrane.

The epimastigotes, due to lack of contrast, could not be readily seen in the cell's cytoplasm after penetration, because of this it was not possible to follow the transformation of the epimastigotes inside the cell by phase technique. However intracellular amastigotes did become evident during incubation and, when they filled the cell, they transformed into trypomastigotes. The process of transformation was followed in Giemsa stained preparations of infected HeLa cells grown on coverslips in test tubes. First the intracellular amastigotes elongated and as elongation continued pyriform epimastigote-like organisms with an anterior free flagellum and a kinetoplast just anterior to the nucleus formed Forms in which the kinetoplast had migrated to the posterior then developed. These forms had a fairly large size and a rounded body and eventually produced small stumpy trypomastigotes. In the cell lines and at the temperature at which we cultured the organism, elongation appeared to be the means of production of trypomastigotes and the organisms appeared to progress through a stage with many epimastigote characteristics.

Occasionally actively motile trypomastigotes in membrane bound vacuoles were seen, either inside the cells (fig. 3A and B) or in the medium (fig. 3C). There was only one organism in each vacuole and there were a number of vacuoles in each cell. The parasites in vacuoles were seen in HEp-2 cells at

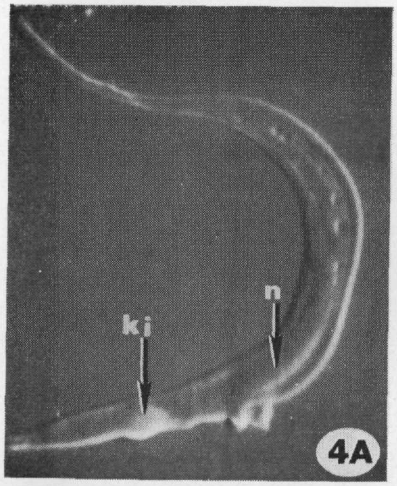


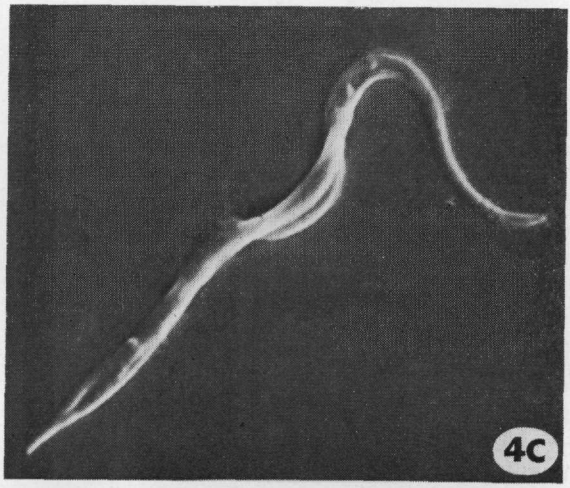
FIGURES 3A–C. Phase contrast photomicrographs of HEp-2 cells with membrane bound vacuoles containing trypomastigotes (trv). The membranes of the vacuoles are clearly visible (see arrows). Each vacuole contains only one actively motile trypomastigote. A membrane enclosed trypomastigote free of any cell is seen in 3C.

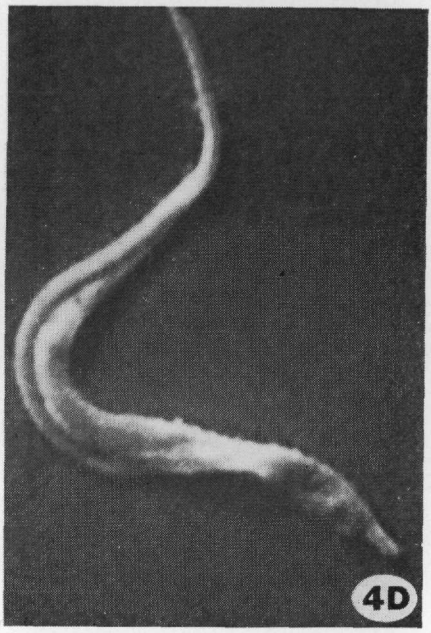
about the fifth day of incubation at 32.5°C. The parasite-containing vacuoles were observed moving around in the cell cytoplasm and sometimes they protruded from the cell. On occasion such vacuoles were observed to be released without loss of the cell's integrity. The usual method of trypomastigote release is by cell rupture. The release of vacuoles containing parasites without cell rupture illustrates the variability of biological systems.

OBSERVATIONS ON THE FINE STRUCTURE OF CELL-CULTURE TRYPOMASTIGOTES OF *Trypanosoma cruzi*

SEM views of the cell-culture trypomastigotes are shown in figure 4A, C, D.







FIGURES 4A–D. SEM images (4A, C–D) and a carbon replica (4B) of cell culture trypomastigote forms of *T. cruzi*. The broad flat form of the parasite usually assumes a C shape (4A–B). The long slender trypomastigote (4C) is in an I shape. Intermediate forms also occur (4D). In 4A, the nuclear (n) and kinetoplast (ki) regions are evident.

Figure 4B is an electron micrograph of a carbon replicated organism. The morphology of these parasites is highly variable and ranges from extremely slender I shaped forms with a pointed posterior (fig. 4C, D) to stouter C shaped forms (fig. 4A, B). Brener (1965; 1969) presented evidence that the slender trypomastigotes, by virtue of their shape and progressive motility, are better adapted to enter cells than stout forms. As the trypomastigote form enters the cell, posterior end first (Dvorak and Schmunis, 1972), the sharply pointed ends which occur on this form would appear to be better suited for penetration than the blunt ends of the broad flat form. The large stumpy forms are usually seen in the mammalian host during the later stages of the infection (Rodriguez and Marinkelle, 1972). As all the forms, from long slender to broad flat and stumpy, appeared in the cell cultures, the process that initiates differentiation cannot be the host immune response although the immune response may select among the differentiated forms.

The texture of the outer surface of these organisms appears to be smooth. However, they may have a more or less granular topography. The granulation is particularly evident in the micrograph of the carbon replicated trypomastigote (fig. 4B). This is probably due to the fact that the transmission electron microscope has a higher resolving power than the scanning microscope. The surface topography of the bounding membranes of the cell culture trypomastigote form of *T. cruzi* appears to be very similar to that of the salivarian trypanosome *T. congolense* and of the epimastigote form of *T. cruzi* (Seed *et al*, 1972; 1973). Internal structures of the scanned broad flat trypomastigotes such as the long bacilliform nucleus and the posteriorly located spherical kinetoplast, are visible (fig. 4A). The flagellar pocket where the flagellum originates is located in the posterior third of the organism. The flagellum runs from there alongside the parasite's body. In some specimens the flagellum, just before reaching the anterior extremity, crosses the body, runs a short distance along the other side and then ends in a moderately long, free flagellum. The flagellum adheres tightly to the body forming a narrow undulating membrane (fig. 4C, D). Disjunction between the flagellum and the body of the parasite was never seen in any of the scanned or carbon replicated organisms. Instead, a shallow groove separating the two was evident.

The flagellum was not actually observed entering the parasite body at the flagellar pocket region in the scanned or carbon replicated trypomastigotes. In freeze-etched trypomastigotes, on the other hand, from which the outer layer of the pellicle was stripped, the entrance of the flagellum into the triangular flagellar pocket was clearly visible (fig. 5A). This is probably an indication that there is, in the cell culture trypomastigotes, a common pellicular layer that envelops both the flagellum and the parasite body. This common layer, which very likely is a surface coat, would be responsible for the hiding from view of the entrance of the flagellum into the flagellar pocket in the various carbon replicas and scanning images of the trypomastigotes.

The point at which the flagellum passes into the flagellar pocket was observed in scanning micrographs of cultured epimastigotes. It is probable that the region of the flagellar pocket would appear similar in the trypomastigotes, although, since the surface coat is probably thicker on the trypomastigotes, the actual point of entrance may not be observed.

The relationship of the body to its locomotory flagellum, noted by Kreier and his co-researchers (Seed *et al*, 1973) in the epimastigote form, was confirmed in this work and it was demonstrated to be similar in trypomastigotes. No disjunction between the parasite's body and the flagellum was seen in either the freeze-fractured, carbon-replicated, or scanned trypomastigotes because the disjunction was covered by the common layer which enveloped both the body and the flagellum. Kreier and his co-investigators were uncertain as to the nature of this common layer although they emphasized that it might represent an additional pellicular layer which was probably removed during the process of specimen preparation for thin sectioning, leaving behind the residue between the flagellum

and the body, termed "macula adherens" by Vickerman (1969). In the trypomastigotes, the common layer seemed to represent the surface coat or at least a portion of it. The common layer was not noted by Maria *et al* (1972) in thin sectioned blood stream forms of *T. cruzi* although a surface coat is clearly evident. Maria and his co-researchers concluded from thin sectioned material that the flagellar membrane was connected to the body pellicle by either a 175 Å thick electron dense zone or a series of fibrous condensations of the desmosome type.

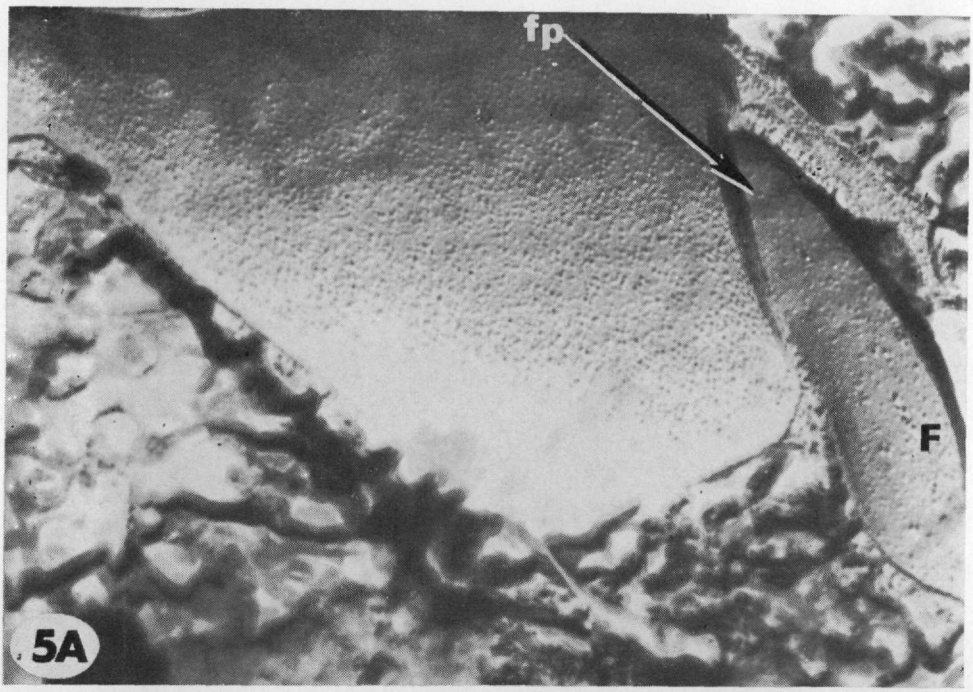
Figure 5B is a freeze-cleaved trypomastigote in which an organism was fractured in two planes. One plane is probably between the inner and the outer hydrophilic layers of the limiting cytoplasmic membrane exposing a large area of the inner face of this membrane. The other is an oblique transverse fracture through the cytoplasm in the nuclear region. This figure provides a particularly good view of various layers of the pellicle complex. The inner face of the cytoplasmic membrane is covered with approximately 6–8 nm particles. This is in contrast to the outer face of the limiting membrane which looks fairly smooth. The outmost layer or the surface coat which was described by Vickerman (1969) and Seed *et al* (1972) in the African trypanosome *T. congolense* is also present in this organism. In most freeze-fractured preparations, however, the surface coat and the outer lamella of the unit membrane were cleaved as one unit. Nevertheless, the presence of the rather thick surface coat is apparent in the lower central position of the figure. The flagellar unit membrane may also have been fractured through the inner hydrophobic region separating ints two hydrophilic layers. The face of the inner layer of the flagellar unit membrane which is oriented toward the outside of the cell is morphologically distinct from the corresponding face of the cytoplasmic membrane. It is covered with granules that are, in general, larger and somewhat flatter than those of the plasma membrane. These granules are almost identical with those of the outer flagellar unit membrane face that is oriented toward the inside of the cell. The common smooth surface layer covers both the parasite flagellum and body in a continuous sheet. Where the oblique transverse fracture passes through the cytoplasm in the nuclear region, the nucleus, nuclear membrane, and an inhomogeneity of the nucleoplasm are revealed. The central, dense finely granular mass of nucleoplasm may be the nucleolus. A number of other investigators (Sanabria 1966, 1968; Brack 1968) have shown in sectioned material that the nucleus of the parasite contains a nucleolus, and that the chromatin material is inhomogeneous. As the nucleus of the freeze-fractured parasite shows the same type of inhomogeneity as does that of the sectioned parasite, the nuclear inhomogeneity is not an artifact of the fixation procedure.

A freeze-cleaved trypomastigote in which the cleavage plane passed through the kinetoplast region is shown in figure 6. In this figure the rounded kinetoplast and the tubular mitochondrion are apparent. The outer surfaces of the two organelles' bounding membranes are almost identical, being only slightly granular. The membranes of the organelles are much less granular than the plasma membrane. These characteristics of the kinetoplast of the trypomastigotes are similar to the characteristics of the epimastigote kinetoplast previously described (Seed *et al*, 1973).

EXPLANATION OF FIGURE 5A AND 5B

FIGURE 5A. Posterior part of a freeze-cleaved trypomastigote form of *T. cruzi*. The stripping of the outer limiting layer exposed the entrance of the flagellum (F) into the flagellar pocket show by the arrow labeled (fp).

FIGURE 5B. Freeze-cleaved cell culture trypomastigote. The cleavage plane passes through the nucleus (n). The outer limiting layer (cm), common to the flagellum and the parasite body, whic his evident in the epimastigote form, is also seen here. The granulation of the inner fracture face of the cytoplasmic membrane (cm-i) and the flagellar unit membrane inner face (fm-i), are very similar to those of the epimastigote. An outer lamella covering the cytoplasmic membrane (cm) is also shown.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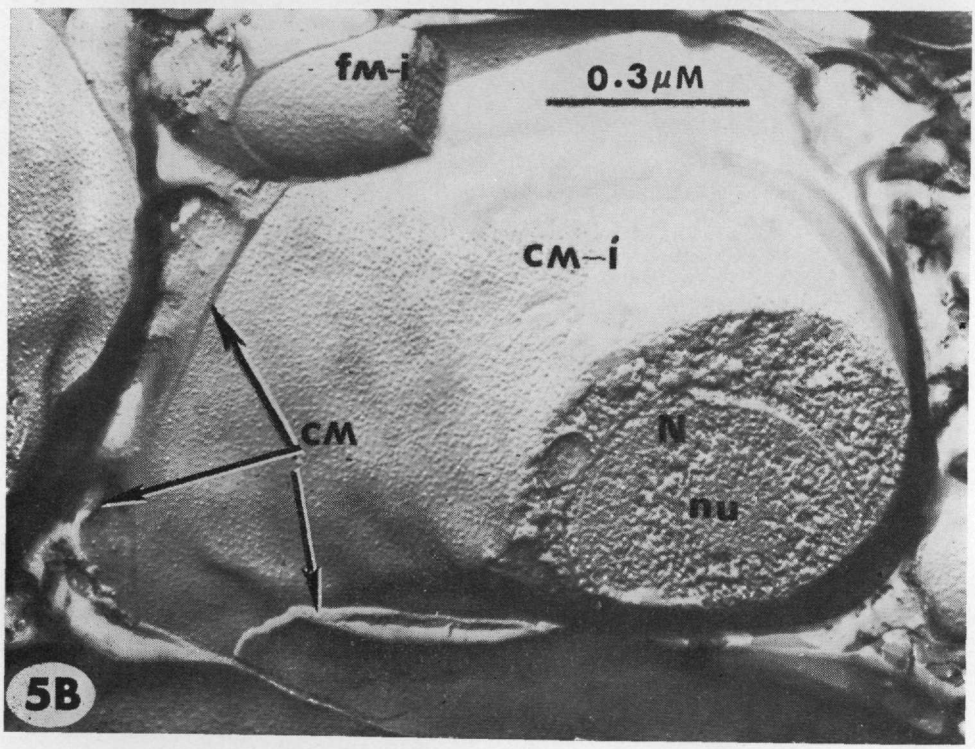
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This study has provided various new insights into the modes of entry, development, release and ultrastructure of *T. cruzi* parasites grown in cell cultures. The scanning electronmicrographs, for example, revealed that penetration of host cells in culture by epimastigotes involves entanglement of the parasite flagellum and host cell filopodia. This observation explains why it is difficult to infect cells in suspension cultures with epimastigotes. In the scanning electron-

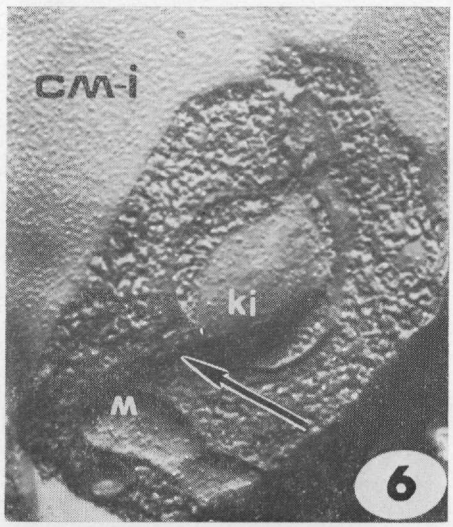

FIGURE 6. Electron micrograph of freeze-cleaved trypomastigote form of *T. cruzi*. The cleavage occurred in two planes: one through the cytoplasmic membrane exposing its inner face (cm-i); the other, a cross fracture through the cytoplasm at the posterior portion of the parasite. As a result of the cleavage the sperhical kinetoplast (ki) and the tubular mitochondrion (m) are revealed. A connection between the two is visilbe (see arrow). The outer faces of the two organelle surfaces appear identical and different from cm-i.

micrographs it also appears as if the parasites penetrate the host cell membrane and do not enter by a phagocytosis type process. Another interesting observation is that there may be a mechanism for release of parasites from cells by a means other than cell rupture. If such a process occures *in vivo* it would permit the parasites to get from cell to cell without causing cell death. We know that many infected individuals remain asymptomatic. A mechanism of parasite dissemination within the body which does not cause cell death would help explain this observation. Perhaps the most interesting possiblity suggested by our morphologic studies is that the surface coat which envelopes the flagellum and the body of the parasite probably also serves to bind them together.

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